# DETECTION OF *YERSINIA PESTIS* BY COMPARISON OF VIRULENCE PLASMID (PYV/PCD)-ASSOCIATED PHENOTYPES IN *YERSINIA* SPECIES*

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## ABSTRACT

*Consumption of meat contaminated with* Yersinia pestis *can cause oropharyngeal plague in humans. Existing microbiological media designed for selective detection of* Y. pestis *in food are not satisfactory for that purpose. Expression of genetic determinants in* Yersinia *species including low calcium response (Lcr), colony size, crystal violet (CV) binding, Congo red (CR) uptake, autoagglutination (AA) and hydrophobicity (HP) were compared. Lcr and CV binding were detectable within 24 h at 37C in* Yersinia enterocolitica *and* Yersinia pseudotuberculosis *but at 48 h in Y. pestis. Colony size, AA, and HP characteristics were expressed in* Y. pseudotuberculosis *and* Y. enterocolitica*, but not in* Y. pestis*. CR uptake in* Y. pestis *was demonstrated only on calcium-deficient CR-magnesium oxalate (CR-MOX) tryptic soy agar but was expressed on both CR-MOX and low-calcium agarose medium for* Y. pseudotuberculosis *and* Y. enterocolitica*. CR-uptake phenotype by* Y. pestis *can be used for the detection of this pathogen in foods.*

## PRACTICAL APPLICATIONS

*Yersinia pestis*, *Yersinia enterocolitica* and *Yersinia pseudotuberculosis* can cause diverse human diseases. *Y. enterocolitica* and *Y. pseudotuberculosis*

* Mention of trade names or commercial products in this publication is solely for the purpose of providing specific information and does not imply recommendation or endorsement by the U.S. Department of Agriculture.

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can cause intestinal distress when consumed in contaminated food. *Y. pestis*, the causative agent of bubonic plague in humans, is also found in food and can cause febrile illness in humans. Thus, food can have a significant role in the dissemination of human plague. There is a concern for risk assessors about the presence of *Y. pestis* in food. A virulence plasmid of 70 kb is directly involved with virulence of these pathogens. Until now, separating *Y. enterocolitica* and *Y. pseudotuberculosis* from *Y. pestis* required sophisticated molecular biology and immunological techniques. This problem was eliminated by analyses of virulence plasmid encoded phenotypes as simple and reliable targets for the rapid detection of *Y. pestis*. These classical microbiological assays should greatly assist both regulatory agencies and the food industry in the detection of *Y. pestis*. Further, these techniques should also greatly assist medical and public health laboratories to identify this pathogen.

## INTRODUCTION

*Yersinia pestis*, *Yersinia enterocolitica* and *Yersinia pseudotuberculosis* can cause diverse human diseases. *Y. enterocolitica* and *Y. pseudotuberculosis* cause gastrointestinal disease characterized by diarrhea and are associated with the consumption of contaminated meat (particularly pork), milk, dairy products, powdered milk, cheese, tofu and raw vegetables (Weagant *et al.* 1998; Nowgesic *et al.* 1999; Bhaduri 2001; Robins-Browne 2001; Nuorti *et al.* 2004). *Y. pestis*, the causative agent of bubonic plague in humans, is highly infectious because the organism can establish pneumonic routes of infection. Although *Y. pestis* has only rarely been implicated as a foodborne pathogen, reports have demonstrated its role in foodborne outbreaks of oro-pharyngeal plague caused by the consumption of inadequately cooked goat and camel meat and bubonic plague caused by slaughtering and skinning domestic animals infected with *Y. pestis* (Christie *et al.* 1980; Arbaji *et al.* 2005; Bin Saeed *et al.* 2005). These incidents showed that domestic animals are susceptible to naturally occurring plague infection and may have a significant role in the dissemination of human plague. Furthermore, the identification of multidrug-resistant strains (Gailmand *et al.* 1997) and the potential use of this pathogen in food by deliberate contamination could cause a plague in large populations in the United States.

At present, the World Health Organization (Dennis *et al.* 1999) recommends the use of brain heart infusion agar (BHA) with sheep blood and MacConkey agar for the isolation of *Y. pestis*. These growth media are suitable for sterile food. However, the isolation of *Y. pestis* in nonsterile food is

complicated by the presence of background flora competing for nutrients and resources of the medium. Thus, numerous colonies grown on these nonselective plates require additional testing for the identification of this pathogen. MacConkey agar possesses a certain degree of selectivity, but the presence of crystal violet (CV) and bile salt restricts the growth of *Y. pestis* (Ber *et al.* 2003). *Y. pestis* strains exhibit slow or no growth *in vitro* on both cefsulodin-irgasan-novobiocin agar and irgasan-nystatin agar (Ber *et al.* 2003) selective media when tested in our laboratory (unpublished data). This may be because of the levels of selective substances used in this media. The colonies formed on selective media require further tests to identify them as *Y. pestis*. These tests are time-consuming, costly and labor intensive because large numbers of presumptive colonies have to be screened.

The chromosomally encoded pigmentation phenotype ($Pgm^+$) refers to the ability of *Y. pestis* to absorb hemin or Congo red (CR) to form dark red colonies on selective agar medium and was used for detection purposes (Surgalla and Beesley 1969). However, this technique is not dependable because of lack of a contrast between the medium and the colonies and often there is a high frequency of spontaneous deletion of Pgm locus (Perry and Fetherston 1997; Lowell *et al.* 2007). Moreover, the occurrence of this phenotypic pigmentation has been found in other pathogens (Perry and Fetherston 1997). Thus, this detection system is critically deficient because of false-positive and false-negative results. Hence, there is no working classical microbiological method for the detection of *Y. pestis*.

All three pathogenic species have been shown to carry a 70-kb virulence plasmid (pYV, also designated as pCD in *Y. pestis*) (Bhaduri 2001; Robins-Browne 2001). Evidence for the direct involvement of this plasmid pYV/pCD in virulence has been described (Perry and Fetherston 1997; Brubaker 2000; Carniel 2000; Skurnik *et al.* 2002). In all three pathogens, carriage of pYV/pCD imparts a calcium-dependent growth phenotype (low calcium response [Lcr]) when cultured at 37C (Bhaduri 2001; Robins-Browne 2001). The pYV/pCD in *Y. enterocolitica* has been correlated with several other phenotypes, including colony morphology/size (size = 1.13 mm), CV binding (dark-violet colony), CR-uptake (red pinpoint colony, size = 0.36 mm), autoagglutination (AA = cells agglutinate) and hydrophobicity (HP = forms clumps), which are expressed at 37C but not at 28C (Bhaduri 2001; Robins-Browne 2001). These well-characterized pYV/pCD-associated virulence determinants had been used for isolation and detection of various serotypes of pYV/pCD-bearing *Y. enterocolitica* in food (Bhaduri and Cottrell 1997; Bhaduri *et al.* 1997).

This study was undertaken to determine whether the phenotypic characteristics of pYV/pCD, including CV binding, colony morphology/size, Lcr, CR-uptake, AA and HP, are expressed in *Y. pestis* and to use

differential expression of these phenotypes for the detection of *Y. pestis* in foods.

## MATERIALS AND METHODS

### Preparation of Media

Brain heart infusion (BHI) broth, BHA and 0.1% peptone water were prepared as recommended by the supplier (Becton-Dickinson, Sparks, MD). Low-calcium (238 μM $Ca^{2+}$) BHI agarose (BHO) was prepared by adding agarose (GibcoBRL, Grand Island, NY) to final concentration of 1.2% to BHI broth supplemented with 0.1% magnesium chloride (Sigma Chemical Co., St. Louis, MO), and CR (Sigma Chemical Co.) was added to BHO at a concentration of 75 μg/mL to prepare CR-BHO as described previously (Bhaduri *et al.* 1990; Bhaduri *et al.* 1991). Calcium-deficient magnesium oxalate agar (MOX) was prepared by adding 20% D-galactose (Sigma Chemical Co.), 0.25 M sodium oxalate (Sigma Chemical Co.) and 0.25 M magnesium chloride (Sigma Chemical Co.) to tryptic soy agar (Becton-Dickinson) (Bhaduri *et al.* 1990) and CR-MOX was prepared by adding 1% CR to MOX as described previously (Bhaduri *et al.* 1991).

### *Yersinia* Strains

A derivative of pYV/pCD-bearing clinical strain of Kurdistan Iran Man (KIM) of *Y. pestis* (KIM 5) lacking the chromosomally encoded pigmentation (CR binding) (Perry and Fetherston 1997) virulence determinants ($Pgm^-$) was used in this study because the native strain leads to confusion of whether the CR binding was coded by Pgm locus or pYV/pCD. Thus, the use of this strain provides an advantage to show specifically pYV/pCD-encoded CR-uptake in *Y. pestis*. Strain Kuma, a derivative of a clinical strain of *Y. pestis* containing a chromosomally encoded pigmentation (CR binding) virulence determinant ($Pgm^+$) but lacking pYV/pCD, was also used to differentiate CR-uptake encoded by the Pgm locus and pYV/pCD, respectively (Perry and Fetherston 1997) and also used as pYV/pCD-less strain. Both *Y. pestis* KIM 5 ($Pgm^-$) and *Y. pestis* Kuma ($Pgm^+$) have been extensively used to study the molecular pathogenesis of this bacterium (Perry and Fetherston 1997; Brubaker 2000; Carniel 2000). Clinical isolates of *Y. pseudotuberculosis* (serotype O:1b; strain PB1/+) and *Y. enterocolitica* (serotype O:3; strain GER) were also used in the current study. The *Y. pestis* and *Y. pseudotuberculosis* strains were kindly provided by Dr. Susan Straley (Department of Microbiology and Immunology, University of Kentucky, Lexington, KY). Only one *Y. pestis* KIM 5 ($Pgm^-$) and *Y. pseudotuberculosis* (serotype O:1b;

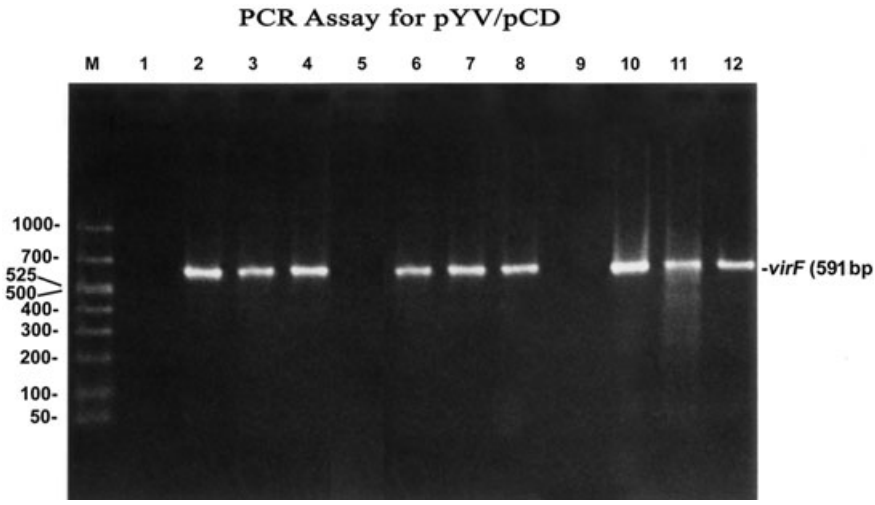


FIG. 1. CONFIRMATION OF PRESENCE OF PYV/PCD IN THE ORIGINAL STRAINS, CELLS IN RED PINPOINT COLONIES AND CELLS IN RED PINPOINT COLONY SURROUNDED BY WHITE BORDER BY PCR ASSAY TARGETING A KEY REGULATORY GENE *VIRF*, WHICH ENCODES A TRANSCRIPTIONAL ACTIVATOR FOR THE EXPRESSION OF PYV/PCD-ENCODED OUTER MEMBRANE C PROTEIN YOP51

The primer pairs (5′-TCATGGCAGAACAGCAGTCAG-3′ and 5′-ACTCATCTTACCATTAA GAAG-3′) for detection of the *virF* gene (430- to 1020- nucleotide region) amplified a 591 base pair (bp) product from the virulence plasmid. Lane M, 50–1000 bp ladder marker; lanes, 1, 5 and 9 showing the absence of 591-bp product in cells from the white borders of *Yersinia enterocolitica*, *Yersinia pseudotuberculosis* and *Yersinia pestis*, respectively; lanes 2, 6, and 10 showing the presence of 591-bp product in the original strains of *Y. enterocolitica*, *Yersinia pseudotuberculosis* and *Y. pestis* respectively; lanes 3, 7 and 11 showing the presence of 591-bp product in cells from the red pinpoint colony of *Y. enterocolitica*, *Y. pseudotuberculosis* and *Y. pestis*, respectively; and lanes 4, 8 and 12 showing the presence of 591-bp product in cells from the red pinpoint colony surrounded by white border of *Y. enterocolitica*, *Y. pseudotuberculosis* and *Y. pestis*, respectively.

strain PB1/+) is available from the worldwide culture collection. *Y. enterocolitica* (serotype O:3; strain GER) was obtained from the culture collection of the Microbial Food Safety Research Unit of the United States Department of Agriculture (USDA) Eastern Research Center (Wyndmoor, PA). *Y. pestis* KIM 5 ($Pgm^-$), *Y. pseudotuberculosis* (serotype O:1b; strain PB1/+) and *Y. enterocolitica* (serotype O:3; strain GER) strains harbored pYV/pCD as confirmed by a PCR assay targeting a key regulatory gene, *virF*, present on pYV/pCD as described by Bhaduri (2003) (Fig. 1 lanes 2, 6 and 10). *Y. pestis* Kuma strain did not show the presence of pYV/pCD by PCR assay. The pYV/pCD-less strains of *Y. pseudotuberculosis* and *Y. enterocolitica* were obtained from large flat colonies that emerged spontaneously from

pYV/pCD-positive cultures growing at 37C on BHO as described by Bhaduri *et al.* (1990). These two strains and Kuma strain were used as negative controls for the expression of pYV/pCD phenotypes.

## Determination of pYV/pCD-Associated Phenotypic Characteristics

The expression of genetic determinants of pYV/pCD in *Y. pestis*, *Y. pseudotuberculosis* and *Y. enterocolitica* was evaluated as follows. The pYV/pCD-bearing cells ($P^+$) and pYV/pCD-less cells ($P^-$) were grown separately in BHI broth for 18 h at 28C without shaking. The cells were diluted to $10^3$ cells per mL (determined by $A_{600}$) in 0.1% peptone water and 100 µL surface plated onto BHA. After 24–48 h of incubation at 37C, the CV (Becton-Dickinson) binding assay was performed by gently flooding the plates with 8 mL of a 100-µg/mL solution of CV for 2 min and then the CV was decanted (Bhaduri *et al.* 1987). The binding of CV to $P^+$ colonies was observed by their dark-violet appearance, while $P^-$ colonies failed to bind the dye and remained white. After performing the CV binding assay the colony size was determined (diameter size in mm) and also the colony morphology was further observed by using a low-magnification (7×) stereomicroscope equipped with a white light source (model ASZ30L3: Bausch & Lomb, Inc., Rochester, NY) (Bhaduri *et al.* 1987; Bhaduri and Cottrell 1997). Hydrophobicity was determined by latex particle agglutination test as described previously (Lachica and Zink 1984; Bhaduri *et al.* 1987). Suspension of latex particles (size: 6.0 µm; Polysciences Inc. Warrington, PA) were diluted in 0.9% saline. Ten µL of this diluted suspension was placed on a slide and mixed with a loop of a colony grown on BHA at 37C for 24–48 h. The $P^+$ cells formed clumps and the $P^-$ cells remained dispersed. The autoagglutination test was determined with Eagle's minimal essential medium supplemented with 10% fetal bovine serum (Weagant *et al.* 1998). Two mL of Eagle's minimal essential medium supplemented with 10% fetal bovine serum in sterile glass tubes was inoculated with a loop of $P^+$ and $P^-$ cells, respectively. Both cultures were incubated for 24–48 h without shaking. The growth of each culture was examined for evidence of bacterial agglutination. The growth of autoagglutination-positive cells consisted of an irregularly edged layer of agglutinated cells that formed a flocculate covering the bottom of the tube. The majority of $P^-$ cells remained in suspension, although some $P^-$ cells had settled to form a smooth round pellet in the center of the bottom of the tube. The $P^+$ and $P^-$ cells were also grown separately in BHI broth for 18 h at 28C without shaking. The cells were then diluted to $10^3$ cells per mL (determined by $A_{600}$) in 0.1% peptone water and as described above and 100 µL surface plated onto BHO and MOX, CR-BHO, and CR-MOX for expression of Lcr and CR-uptake, respectively. The cells were incubated at 37C for 24–48 h to assess Lcr by the appearance of pinpoint

colony (size 0.36 mm) and CR-uptake (red pinpoint colony size of 0.36 mm) (Bhaduri *et al.* 1990; Bhaduri *et al.* 1991).

## RESULTS AND DISCUSSION

The flooding of plates with of pre-grown colonies of $P^+$ strains on BHA at 37C after incubation for 24 h for *Y. pseudotuberculosis* and *Y. enterocolitica* and for 48 h for *Y. pestis* with CV solution (100 µg per mL) showed that $P^+$ cells from all three *Yersinia* species produced dark-violet colonies (Table 1; Fig. 2 [i] A). The $P^-$ colonies did not bind CV and remained white (Fig. 2 [i] B). The assay can effectively identify individual pYV/pCD-bearing colonies from a mixed culture of $P^+$ and $P^-$ strains as reported previously (Bhaduri *et al.* 1987; Bhaduri *et al.* 1991).

The colony morphology/size of $P^+$ cells of *Y. enterocolitica* and *Y. pseudotuberculosis* were smaller (1.13 mm in diameter) (Fig. 2 [ii] A) than

TABLE 1.
COMPARISON OF SELECTED PHENOTYPIC EXPRESSION OF PYV/PCD-BEARING *YERSINIA ENTEROCOLITICA, YERSINIA PSEUDOTUBERCULOSIS* AND *YERSINIA PESTIS*

| Organism* | Strain | CM† | CV Binding‡ | Lcr§ | CR-uptake¶ | AA** | HP†† | Plasmid‡‡ |
|---|---|---|---|---|---|---|---|---|
| *Y. enterocolitica* | GER | + | + | + | + | + | + | + |
| *Y. enterocolitica*-C | GER | – | – | – | – | – | – | – |
| *Y. pseudotuberculosis* | PB1/+ | + | + | + | + | + | + | + |
| *Y. pseudotuberculosis*-C | PB1/+ | – | – | – | – | – | – | – |
| *Y. pestis* | KIM 5 | – | + | + | + | – | – | + |
| pYV/pCD-less *Y. pestis* | Kuma | – | – | – | – | – | – | – |

* The pYV/pCD-negative strains of *Y. enterocolitica*, and *Y. pseudotuberculosis*, are designated as C (cured).

† CM, colony morphology. The $P^+$ cells appeared as small colonies (1.13 mm in diameter) as compared to larger $P^-$ colonies (2.4 mm in diameter) on BHA.

‡ CV binding, crystal violet binding. The $P^+$ cells appeared as small dark-violet colonies and the $P^-$ cells showed large white colonies on BHA.

§ Lcr, low calcium response/calcium-dependent growth. $P^+$ cells appeared as pinpoint colonies (0.36 mm in diameter) and $P^-$ cells appeared large colonies (1.37 mm in diameter) on low-calcium CR-BHO (238 µM $Ca^{2+}$) and calcium-deficient CR-MOX.

¶ CR-uptake, congo red-uptake. The $P^+$ cells appeared as red pinpoint colonies (0.36 mm in diameter) and the $P^-$ cells appeared large white or light-orange colonies (1.13 mm in diameter) on calcium-deficient CR-MOX or low-calcium CR-BHO (238 µM $Ca^{2+}$).

** AA, autoagglutination. The $P^+$ cells agglutinated. The $P^-$ cells remained dispersed.

†† HP, hydrophobicity by latex particles. The $P^+$ cells formed clumps showing hydrophobicity. The $P^-$ cells remained dispersed.

‡‡ Plasmid, presence of 70-kb pYV/pCD by PCR assay.

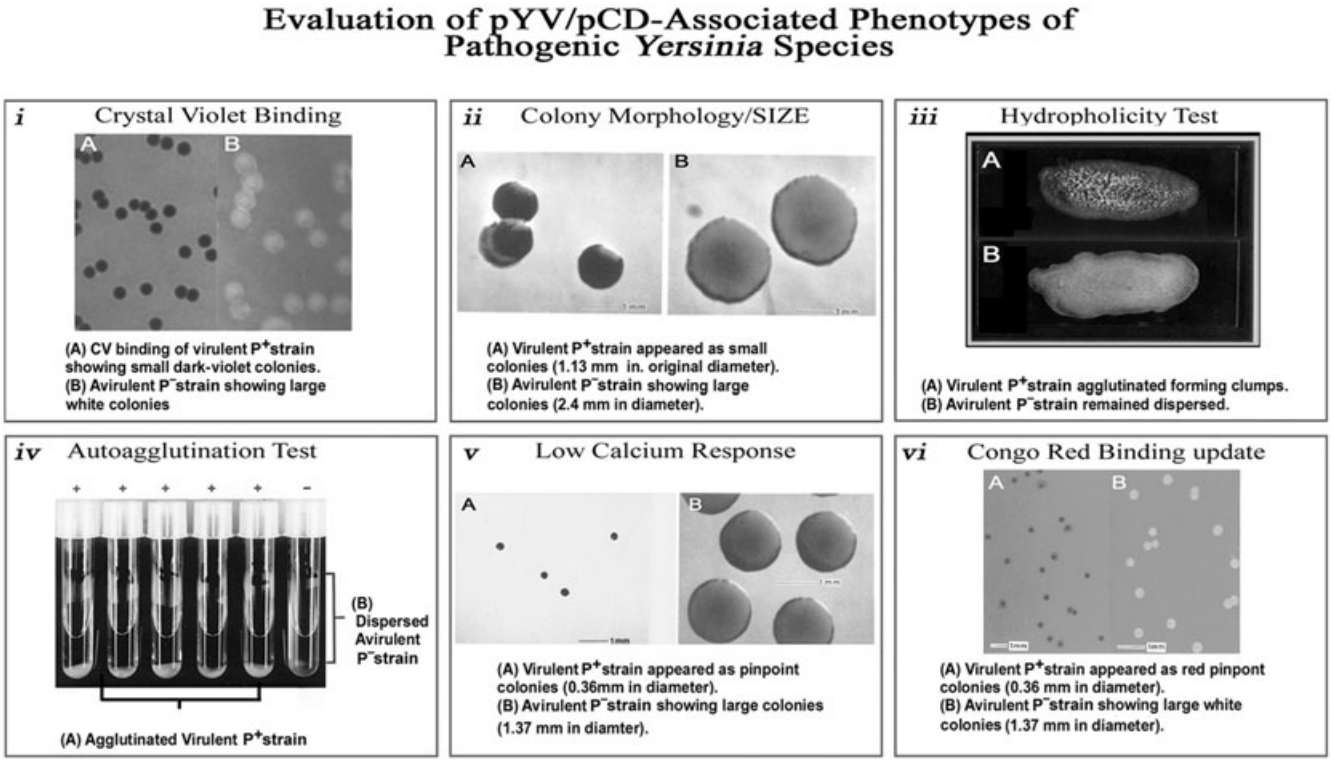


FIG. 2. DEMONSTRATION OF PYV/PCD-ASSOCIATED PHENOTYPES OF YERSINIA SPECIES
The pYV/pCD-bearing cells are designated as virulent $P^+$ strain; pYV/pCD-negative cells are designated as avìrulent $P^-$ strain.

corresponding $P^-$ cells (2.4 mm in diameter) (Fig. 2 [ii] B) when grown on BHA at 37C (Table 1) as reported previously (Bhaduri *et al.* 1990), whereas pYV/pCD-bearing colonies (KIM 5) and pYV/pCD-less colonies (Kuma) of *Y. pestis* were approximately the same size (1.3–1.4 mm; SD $\pm$ 0.11) (Table 1). Colony morphologies of the CV-positive and CV-negative strains of *Y. enterocolitica*, *Y. pseudotuberculosis* and *Y. pestis* were examined further by using a low-magnification stereomicroscope. The CV-postive strains of *Y. enterocolitica* and *Y. pseudotuberculosis* formed small, convex, shiny, dark opaque colonies, whereas the CV-negative strains of *Y. enterocolitica* and *Y. pseudotuberculosis* formed shiny, translucent, flat colonies (Bhaduri *et al.* 1990). When cells of pYV/pCD-bearing KIM 5 and pYV/pCD-less (Kuma) of *Y. pestis* strains are grown under similar conditions, neither of the strains showed these colony morphology characteristics. At present time, it is not clear why this colony morphology characteristic was not expressed in *Y. pestis* even though the optimum growth temperature of *Y. pestis* is similar to that of *Y. enterocolitica* (Skurnik *et al.* 2002).

Hydrophobicity by latex particle agglutination was positive for *Y. enterocolitica* and *Y. pseudotuberculosis* (Fig. 2 [iii] A) but negative for *Y. pestis* when pre-grown cells on BHA at 37C were examined (Table 1). The autoagglutination test in tissue culture was positive for *Y. enterocolitica* and *Y. pseudotuberculosis* (Fig. 2 [iv] A), but the *Y. pestis* culture failed to autoagglutinate (Table 1) at 37C. In both HP and AA tests, $P^-$ strains were negative (Fig. 2 [iii] B and [iv] B) (Table 1). The explanation for the absence of expression of these two phenotypic characteristics under the conditions described above in *Y. pestis* may be because of the fact that pYV/pCD does not encode YadA in *Y. pestis*, a key surface antigen of *Y. enterocolitica* that contributes to HP and AA or structural/regulatory variability of pYV/pCD (Nesbakken *et al.* 1987; Perry and Fetherston 1997; Brubaker 2000; Carniel 2000). It is necessary to use fresh media to express these phenotypic characteristics of pYV/pCD.

The $P^+$ strains of *Y. enterocolitica*, *Y. pseudotuberculosis* and *Y. pestis* cells formed pinpoint colonies (0.36 mm in diameter) showing Lcr phenotype (Fig. 2 [v] A) on both BHO and MOX at 37C by 24 h and by 48 h, respectively (Table 1). The size and colony morphologies of each $P^+$ strain when grown on BHO and MOX showed identical expression of Lcr under these two conditions (Table 1). The $P^-$ cells from each respective strain formed much larger colonies (1.37 mm in diameter) (Fig. 2 [v] B). The difference in expression time of CV binding and Lcr in *Y. pestis* may provide a tool for the detection of this pathogen. The negative response to colony morphology, HP and AA also provide another tool for differentiating *Y. pestis* from *Y. enterocolitica* and *Y. pseudotuberculosis*.

TABLE 2.
EFFECT OF MEDIA ON CONGO RED-UPTAKE IN PYV/PCD-BEARING *YERSINIA ENTEROCOLITICA*, *YERSINIA PSEUDOTUBERCULOSIS* AND *YERSINIA PESTIS*

| Organism* | Strain | CR-BHO | CR-MOX |
|---|---|---|---|
| *Y. enterocolitica* | GER | + | + |
| *Y. enterocolitica*-C | GER | – | – |
| *Y. pseudotuberculosis* | PB1/+ | + | + |
| *Y. pseudotuberculosis*–C | PB1/+ | – | – |
| *Y. pestis* | KIM5 | – | + |
| pYV/pCD-less *Y. pestis* | Kuma | – | – |

* The pYV/pCD-negative strains of *Y. enterocolitica* and *Y. pseudotuberculosis* are designated as C (cured).
CR-BHO, Low-calcium (238 μM $Ca^{2+}$); CR-MOX, calcium deficient.

When $P^+$ and $P^-$ strains were cultivated at 37C for 24–48 h on CR-BHO and CR-MOX the $P^+$ cells of *Y. enterocolitica* and *Y. pseudotuberculosis* showed CR-uptake as red pinpoint colonies (0.36 mm in diameter) (Fig. 2 [vi] A) on both CR-BHO and CR-MOX at 24 h, whereas CR-uptake of *Y. pestis* was insignificant compared to that of *Y. enterocolitica* and *Y. pseudotuberculosis* on CR-BHO (Tables 1 and 2). Increasing the concentration of CR in BHO to 100 μg/mL, 150 μg/mL and 200 μg/mL did not increase the color intensity of *Y. pestis* $P^+$ colonies. On the basis of color contrast between the medium and colonies, CR-MOX showed appropriate conditions for CR-uptake in the *Y. pestis* $P^+$ strain (Table 2) cultivated at 37C for 48 h. The $P^-$ cells from each respective strain failed to bind the dye (Tables 1 and 2) and formed much larger white or light-orange colonies (1.37 mm in diameter) (Fig. 2 [vi] B). Moreover, *Y. pestis* Kuma with chromosomally encoded pigmentation virulence determinants ($Pgm^+$) but lacking pYV/pCD failed to bind CR on CR-BHO and CR-MOX under these same conditions, forming larger white or light-orange colonies (1.37 mm in diameter) as described earlier (Table 2). A number of derivatives of clinical strains of *Y. pestis* (CDC A1122, CO99.3015, Yokohama, P12, D1, D3, D5, D7, D9, D13 and D17) containing chromosomally encoded pigmentation (CR binding) virulence determinants ($Pgm^+$) but lacking the pYV/pCD (Perry and Fetherston 1997) were also used to show absence of pYV/pCD-encoded CR-uptake by plating them on CR-MOX. These strains did not bind CR and thus confirmed that the CR-uptake is expressed by pYV/pCD. These observations indicate that the CR-uptake in *Y. pestis* grown on CR-MOX is independent of chromosomally encoded pigmentation virulence determinants ($Pgm^+$) and that the Pgm locus is not expressed under this condition. This phenotype type is encoded by pYV/pCD only on

calcium-depleted medium. The calcium concentration in CR-BHO (234 μM) is relatively low, whereas in CR-MOX, sodium oxalate is used to sequester the calcium, making the medium calcium deficient (Bhaduri *et al.* 1990). Thus, the CR-uptake in *Y. pestis* is more dependent on calcium depletion than that of *Y. enterocolitica* and *Y. pseudotuberculosis*. Therefore, specific CR-uptake on CR-MOX by *Y. pestis* can be used to differentiate *Y. pestis* from *Y. enterocolitica* and *Y. pseudotuberculosis*.

This would provide diagnostic value as follows: the suspected food samples are plated on CR-BHO and CR-MOX. If the colonies show CR-uptake only on CR-MOX at 37C after 48 h of cultivation, then those $CR^+$ colonies can be identified as *Y. pestis* strains. This technique will enhance the detection of *Y. pestis* strains in the presence of competing microflora through the selection of media and incubation times. The $CR^+$ *Y. pestis* clones can be further confirmed by PCR targeting *Y. pestis* specific plasmid encoded plasminogen activator gene (Loiez *et al.* 2003).

Another characteristic feature of the CR-uptake in *Y. pestis* is the appearance of a white opaque circumference around the red center after 72 h of incubation at 37C as reported previously in *Y. enterocolitica* (Bhaduri *et al.* 1991). The presence of pYV/pCD at the red center (Fig. 1 lanes 3, 7 and 11; lanes 4, 8 and 12) and the absence in the white border (Fig. 1 lanes 1, 5 and 9) was confirmed by PCR assay as reported in *Y. enterocolitica* (Bhaduri *et al.* 1991). The white border around red center is because of loss of pYV/pCD and the concomitant disappearance of the associated CR-uptake phenotypic characteristic. This colony characteristic can also be used as a detection parameter of *Y. pestis* (Bhaduri *et al.* 1991; Bhaduri and Cottrell 1997). To show the specificity of CR-uptake by *Y. pestis* on CR-MOX several species of bacteria including a number of foodborne pathogens were tested (data not shown). These species did not form red pinpoint colonies as well as formation of a white border around the center under the same conditions on CR-MOX (data not shown) showing specificity of this phenotype in *Y. pestis* as reported previously with *Y. enterocolitica* (Bhaduri *et al.* 1991). This method of detection was applied to identify *Y. pestis* in sterile raw ground beef (RGB) artificially contaminated with this pathogen. Ten grams of irradiated sterile RGB was inoculated with 1 mL of a $10^5$ CFU/mL suspension of *Y. pestis* in a stomacher bag. The bags were loosely sealed with tape to permit ambient air exchange. The samples were then incubated at 4C for 24 h. Three samples of RGB were diluted with 0.1% peptone water in 10-fold serial increments, stomached for 2 min, 0.05 mL surface-plated on CR-MOX agar and the plates incubated at 37C for 24 h. The appearance of red pinpoint colonies on agar plates indicated pYV/pCD-bearing *Y. pestis* from RGB. Thus, this verification showed that CR-uptake on CR-MOX by *Y. pestis* provides a classical microbiological method for detection of this pathogen from food.

In conclusion, of the six pYV/pCD-associated phenotypes examined, only three phenotypes (Lcr, CR-uptake and CV binding) were expressed in *Y. pestis*, while all six properties were expressed in *Y. enterocolitica* and *Y. pseudotuberculosis*. The specific CR-uptake of *Y. pestis* in the calcium-deficient medium provides a screening medium to detect and to differentiate this pathogen from *Y. enterocolitica* and *Y. pseudotuberculosis*. This method is also applicable to food. Moreover, the delayed expression of Lcr and CV binding and the nonexpression of colony morphology, HP and AA provide a diagnostic tool for differentiating *Y. pestis* from *Y. enterocolitica* and *Y. pseudotuberculosis*. Thus, a combination of these six pYV/pCD-associated phenotypes expression provides the means to identify *Y. pestis* colonies in clinical samples, animals and food.

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